Tyrosine Kinase Inhibitor Gefitinib Enhances Topotecan Penetration of Gliomas

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Abstract

Gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, increases brain parenchymal extravascular fluid (ECF) accumulation of topotecan, a substrate of the ATP-binding cassette (ABC) transporters P-glycoprotein (Pgp/MDR-1) and breast cancer resistance protein (BCRP/ABCG2). The effect of modulating these transporters on topotecan penetration in gliomas has not been thoroughly studied. Thus, we performed intracerebral microdialysis on mice bearing orthotopic human gliomas (U87 and MT330) and assessed topotecan tumor ECF (tECF) penetration and the effect of gefitinib on topotecan tECF penetration and intratumor topotecan distribution. We found that topotecan penetration \( P_{\text{tumor}} \) of U87 was 0.96 ± 0.25 \((n = 7)\) compared with that of contralateral brain \( P_{\text{contralateral}} \) (0.42 ± 0.11, \( n = 5; P = 0.001)\). In MT330 tumors, \( P_{\text{tumor}} \) (0.78 ± 0.26, \( n = 6) \) and \( P_{\text{contralateral}} \) (0.42 ± 0.11, \( n = 5) \) also differed significantly \((P = 0.013)\). Because both tumor models had disrupted blood-brain barriers and similar \( P_{\text{tumor}} \) values, we used U87 and a steady-state drug administration approach to characterize the effect of gefitinib on topotecan \( P_{\text{tumor}} \). At equivalent plasma topotecan exposures, we found that \( P_{\text{tumor}} \) after gefitinib administration was lower. In a separate cohort of animals, we determined the volume of distribution of unbound topotecan in tumor \( V_{\text{itumor}} \) and found that it was significantly higher in groups receiving gefitinib, implying that gefitinib administration leads to a greater proportion of intracellular topotecan. Our results provide crucial insights into the role that transporters play in central nervous system drug penetration and provide a better understanding of the effect of coadministration of transporter modulators on anticancer drug distribution within a tumor.

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Introduction

Most central nervous system (CNS) tumors, particularly gliomas, are resistant to chemotherapy (1). Drug resistance in brain tumors is not well understood but may involve poor tumor cell exposure (2), which can be caused by several mechanisms. One important mechanism involves active drug efflux from the endothelial cells forming the blood-brain barrier (BBB) by specific ATP-binding cassette (ABC) transporters (3). The irregular distribution of tumor vessels, the absence of lymphatics, and high oncotic pressure can also impede drug penetration to tumor cells distant from vessels (2). Further, some tumor cells can actively export xenobiots via ABC transporters at the cell membrane (4). Intracellular drug exposure is crucial to the effectiveness of agents such as methotrexate (antimetabolite), paclitaxel (tubulin inhibitor), and the camptothecins (topoisomerase I inhibitors), to which cells overexpressing specific ABC transporters acquire resistance (5–7).

The semisynthetic camptothecin analogue topotecan is active against xenografted human CNS tumors (8, 9) but shows little efficacy against high-grade gliomas in clinical trials (10). Poor drug penetration may contribute to this outcome. We and others observed that topotecan penetration of brain parenchyma is restricted by at least two ABC transporters: breast cancer resistance protein (BCRP/ABCG2) and P-glycoprotein (Pgp/MDR1/ABCB1; refs. 11, 12). Further, we found that the tyrosine kinase inhibitor (TKI) gefitinib increased topotecan penetration into the brain extravascular fluid (ECF; refs. 11, 13). Gefitinib, an epidermal growth factor receptor inhibitor, also increased the bioavailability of oral irinotecan in mice (14) and humans (15) and reversed tumor cell resistance to topotecan and irinotecan (7, 16), likely via inhibition of BCRP and Pgp (6, 7). Several other TKIs are modulators of ABC transporters and have been studied, in combination with transporter-substrate chemotherapy agents, to overcome tumor drug resistance or to increase drug bioavailability (17–20).

The effect of ABC transporter inhibition on drug penetration of orthotopic brain tumor models is unknown. The BBB remains functionally intact at the margins of invasive brain tumors, where malignant cells migrate through vessel co-option to adjacent tissues (21, 22); therefore, inhibitors of active drug transporters could potentially improve CNS drug delivery to orthotopic brain tumor models.
efflux at the BBB may increase drug concentration in the tumor ECF (tECF; ref. 11). Efflux transporters have also been found in the neovascularature of the glioma tumor bulk (23, 24). However, because tumor-secreted angiogenic molecules (e.g., vascular endothelial growth factor) increase the permeability of vessels within the tumor bulk (25), it is unclear whether the activity of efflux transporters (and their inhibitors) is significant in these hyperpermeable tumor regions. Finally, if the tumor itself expresses ABC transporters, an inhibitor could act at the tumor cell level to increase topotecan intracellular penetration. Here, we used a combined microdialysis-tumor homogenate technique to compare the distribution of topotecan in the tECF and cells of orthotopic glioma xenografts in the presence versus absence of the pump inhibitor gefitinib. We hypothesized that gefitinib would increase intracellular drug accumulation in ABC transporter-expressing tumors with a permeable BBB by modulating cellular drug efflux.

Materials and Methods

Tumor lines
Two human glioma cell lines were used: U-87 MG (U87) and MT330. The U87 model has been extensively described (26). MT330 was created from a WHO grade 4 glioma sample obtained from a patient at University Methodist Hospital (Memphis, TN) as part of an Institutional Review Board–approved protocol. Both tumor lines, modified to stably express firefly luciferase (27), were donated by Dr. Andrew Davidoff (St. Jude Children’s Research Hospital; Memphis, TN).

Orthotopic tumor xenografts
Animal procedures were approved by the St. Jude Animal Care and Use Committee. CD1 athymic nude mice (8–12 wk old; Charles River Laboratories) were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine and injected with 5 × 10⁵ U87 or MT330 cells suspended in 50 μL Matrigel (Becton Dickinson). With the guidance of a mouse brain atlas (28), cells were injected stereotactically (David Kopf Instruments) into the right caudate putamen.

Tumor growth was monitored by bioluminescence (IVIS-100 imaging system, Xenogen; ref. 29). Briefly, mice were injected i.p. with 120 mg/kg D-luciferin firefly (potassium salt; Caliper Lifesciences) and imaged under isoflurane anesthesia after 5 minutes. Tumor vascular permeability was assessed by contrast-enhanced magnetic resonance imaging (CE-MRI; see Supplementary Data for details).

Topotecan penetration of tECF
The concentration-time profiles of unbound drugs in tissue ECF can be characterized by microdialysis sampling coupled with an analytic method (30). We used this technique to measure the penetration of unbound topotecan lactone into tumor or brain ECF after a single iv. injection of topotecan, as previously described (31). Briefly, a microdialysis guide cannula (MD-2255, Bioanalytical Systems) was inserted through the skull and attached to a microdialysis probe (MBR-1-5, Bioanalytical Systems) inserted through the probe membrane was allowed to equilibrate for 1 hour. Topotecan (4 mg/kg) was then administered, and dialysate samples were collected every 18 minutes. Samples were analyzed for topotecan lactone and carboxylate with an automated microbore high-performance liquid chromatography (HPLC) fluorescence detection system until concentrations were unmeasurable (31). Probe recovery was calculated by retrodialysis as previously described (31). Using a previously described limited sampling strategy (13), we bled each mouse from the retro-orbital plexus 15, 60, and 180 minutes after the i.v. injection to assay plasma topotecan lactone.

The unbound plasma topotecan concentration was calculated on the basis of a previous study (13), showing a 27% unbound fraction in CD1 nude mice. Unbound plasma topotecan levels were modeled by maximum a posteriori probability Bayesian estimation with ADAPT 5 software (Biomedical Simulations Resource; ref. 32). The prior parameter distribution was calculated by nonlinear mixed-effects modeling (NONMEM version VI; ref 33) in an independent group of mice receiving the same topotecan dose, as previously described (11).

The simulated concentration-time data from the ADAPT 5 model estimates were integrated to obtain the area under the concentration-time curve defining plasma exposure to unbound topotecan lactone (AUCu,plasma). Similarly, unbound drug exposure in the dialyzed tissue (brain or tumor) ECF (AUCu,tissueECF) was determined in each mouse (after correcting for probe recovery as previously described; ref. 31) by three-compartment analysis of combined plasma and tissue topotecan lactone pharmacokinetic data (11). Finally, the extent of penetration of the targeted tissue by unbound topotecan lactone (Ptissue) was calculated as the tissue (brain or tumor) ECF-to-plasma AUC ratio (34):

\[ P_{\text{tissue}} = \frac{\text{AUC}_{\text{tissueECF}}}{\text{AUC}_{\text{plasma}}} \]

The probe location was confirmed histologically at the end of all microdialysis experiments. In studies targeting tumor tissue, animals that had the probe outside the tumor were excluded from the analysis.

Western blots and immunohistochemistry for ABC transporters
Western blots of U87, MT330, and control cell lysates (Saos-2, BCRP, LLC-PK1-MDR1, LS180-MRP1, and Saos-2-MRP4, stably transfected with BCRP, Pgp, MRP1, and MRP4, respectively) were performed following previously published methods (35). Membranes were incubated with 1:400 anti-BCRP (Bxp-21; Kamiya Biomedical), 1:400 anti-Pgp (C219), 1:1,000 anti-MRP1

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Effect of gefitinib on topotecan tECF penetration

We maintained a constant (steady-state) topotecan concentration in plasma \( (C_{ss,plasma}) \) to evaluate the real-time effect of a single oral dose of gefitinib (200 mg/kg) on the topotecan tECF penetration. A topotecan-loaded mini-osmotic pump (Alzet model 2001D; Durect) was s.c. implanted in the CD1 nude mice; its ability to maintain a 100 ng/mL plasma topotecan lactone concentration for 24 hours had been confirmed in a group of five wild-type FVB mice (data not shown). To achieve a plasma topotecan concentration \( C_{ss,plasma} \) of >100 ng/mL (bound + unbound) in U87 tumor-bearing mice, we loaded the pump with 4 mg/mL topotecan in sterile water, which was released at 25 \( \mu \)g/h for 24 hours.

For microdialysis studies, the probe recovery was calculated by retrodialysis before the pharmacokinetic experiment. Then, the probe was washed by perfusion for 1 hour with aCSF at 2 \( \mu \)L/min. Mice were then anesthetized with isoflurane, a topotecan-loaded pump previously primed in 0.9% saline (3 h, 37°C) was surgically inserted under the skin of the back, and the incision was sutured. The aCSF flow rate was then reduced to 0.5 \( \mu \)L/min, and dialysate samples were collected overnight every 18 minutes, as described above. The next morning (12–14 h after pump insertion), a plasma sample was obtained to characterize unbound \( C_{ss,plasma} \) and a 200 mg/kg gefitinib dose was administered orally as previously described (13). Dialysate sample collection and analysis continued for 8 to 10 hours, and plasma samples were obtained retro-orbitally 4, 6, and 8 hours after gefitinib administration. The \( C_{ss} \) of unbound topotecan lactone in tECF \( (C_{ss,tECF}) \) was calculated before (mean concentration of dialysate samples collected 6–14 h after pump insertion) and after (mean concentration of dialysate samples collected 6–8 h after gefitinib) the gefitinib dose. The steady-state penetration of tECF \( (P_{ss,tumor}) \) by unbound topotecan lactone was calculated as the \( C_{ss,tECF}/C_{ss,plasma} \) ratio (34).

Intratumor distribution of topotecan

To determine the proportion of drug either entrapped in the intracellular compartment or nonspecifically bound to tumor tissue components, in relation to the unbound drug fraction in the tECF, we applied the "unbound drug volume of distribution in tumor" \( (V_{u,tumor}) \) parameter, adapted from Wang and Welty (36). \( V_{u,tumor} \) describes the relationship, at the steady state, between the total drug concentration in the tumor (assayed in homogenized tissue) and the unbound drug concentration in tECF (calculated by microdialysis). \( V_{u,tumor} \) is measured in mL/g tumor:

\[
V_{u,tumor} = \frac{A_{tumor} - V_{tot,blood} \times C_{tot,blood}}{C_{ss,tECF}}
\]

where \( A_{tumor} \) is the total quantity of drug per gram of tumor homogenate (including blood present in the tumor), \( V_{tot,blood} \) is the volume of blood per gram of tumor, \( C_{tot,blood} \) is the total concentration of drug in blood, and \( C_{ss,tECF} \) is the measured concentration of unbound drug in the tECF.

We obtained experimental \( A_{tumor} \) and \( C_{tot,blood} \) data by performing studies in additional groups of mice bearing U87 tumors using timing similar to that described above. One group, TPT25 \( (n = 8) \), received pump-infused topotecan at a constant rate of 25 \( \mu \)g/h. These animals were sacrificed 8 to 12 hours after insertion of the pump (at steady state), and blood and tumor tissues were harvested for HPLC drug assays, performed as already published (37). The effect of gefitinib on the \( V_{u,tumor} \) parameter was assessed in two additional groups of U87 tumor-bearing mice. The TPT25-GEF group \( (n = 7) \) received topotecan at 25 \( \mu \)g/h and received oral gefitinib (200 mg/kg) 8 hours before sacrifice. The TPT12-GEF group \( (n = 6) \) received an adjusted dose of topotecan (12.5 \( \mu \)g/h) to achieve systemic exposure \( (C_{ss,plasma}) \) comparable with that in the TPT25 group and received oral gefitinib (200 mg/kg) 8 hours before sacrifice. Tumors were immediately frozen and stored at −80°C. For drug analysis, tumors were weighed and homogenized ultrasonically (CP-50, Cole-Parmer) at 4°C in water (10 \( \mu \)L/mg of tumor). The tumor suspension (200 \( \mu \)L) was mixed vigorously with 800 \( \mu \)L of cold methanol and centrifuged for 10 minutes at 14,000 rpm at 4°C. The supernatant was filtered in Spin-X Centrifuge filter tubes (Costar) at 14,000 rpm at 4°C for 10 minutes. Filters were stored at −80°C for HPLC assay.

To estimate the \( V_{u,tumor} \) parameter in the TPT25 and TPT25-GEF groups, we used the mean \( C_{ss,tECF} \) values obtained in the previous steady-state microdialysis experiments. The value \( V_{blood} \) for intracranial U87 tumors in mice was obtained from the literature (4.1%; ref. 38). In the TPT12-GEF group, we performed a new set of microdialysis experiments \( (n = 3) \) to obtain the \( C_{ss,tECF} \) value (as described above) in animals bearing U87 tumors and colocalized microdialysis cannulae; in this group, gefitinib (200 mg/kg, oral) was administered 30 minutes before insertion of a pump dispensing 12.5 \( \mu \)g/h of topotecan. Dialysate samples were collected 8 to 10 hours after insertion, and a blood sample was obtained 8 hours after gefitinib administration.

Intracellular accumulation of topotecan in vitro

Cells \( (5 \times 10^5 \) per well in 2 mL medium) were incubated overnight in six-well tissue culture plates (Becton Dickinson), and 2 mL of medium containing topotecan (final concentration, 1 \( \mu \)mol/L) with or without gefitinib (final concentration, 10 \( \mu \)mol/L) were then added. After incubation at 37°C for 1, 5, or 10 minutes, the medium was rapidly aspirated to terminate drug accumulation, and the wells were washed with ice-cold PBS \( (2 \times 5 \text{ mL}) \). Ice-cold water was added (500 \( \mu \)L/well), and cell suspensions were transferred to microcentrifuge tubes on ice. Samples were suspended ultrasonically, and 100 \( \mu \)L of suspension were added to 400 \( \mu \)L of cold methanol. The mixture was mixed vigorously and centrifuged for 10 minutes at 14,000 rpm at 4°C. The supernatant was filtered in a Costar Spin-X...
Centrifuge filter tube at 14,000 rpm at 4°C for 3 minutes. Filtrates were stored at −80°C for HPLC assay. Samples were analyzed by HPLC as described previously (31).

**Drug sensitivity**

The antitumor activity of topotecan, gefitinib, and their combinations was determined in vitro in U87 and MT330 tumor models as previously described (6). After 4-hour incubation, the drug-containing medium was replaced with fresh medium and the proportion of viable cells was determined 4 days later.

**Statistics**

Aggregate data are presented as mean ± SD. Paired t test was used to compare variables in animals before and after a treatment (i.e., gefitinib). Student's t test and the Mann-Whitney

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**Figure 1.** Morphology and growth of U87 and MT330 tumor models. A, histologic appearance of the xenografts (H&E stain). Dotted yellow lines indicate the tumor margin. B, tumor bioluminescence signal versus time. Points, mean of 5 to 15 values; bars, SD. Representative bioluminescence pictures are shown.
test (for nonnormally distributed data) were used for nonpaired
comparisons of two groups. For the comparison of more
than two groups, a one-way ANOVA was performed followed
by a post hoc \( t \) test with Bonferroni correction.

**Results**

**Glioma morphology and growth in vivo**

We studied drug penetration of gliomas by orthotopically
implanting the most frequently used glioma cell line (U87)
and a new low-passage primary tumor line (MT330). The
tumor cells engrafted in 100% (58 of 58, U87) and 85%
(17 of 19, MT330) of inoculated animals. U87 tumors,
as previously described (39), were round and noninfiltrative
(Fig. 1A); had profuse, uniform vascularization; and were easily
dissected from surrounding brain under a dissecting micro-
scope. In contrast, MT330 tumors infiltrated contiguous
brain parenchyma, had irregular edges, had inhomogeneous
core vasculature, and could be dissected only by using a
bioluminescence-guided technique.

Tumor growth, as measured by increased bioluminescence
signal (29), was also dissimilar (Fig. 1B): U87 tumors were >50
mm\(^3\) (assessed by terminal dissection) 2 to 3 weeks after im-
plantation, and most animals showed neurologic signs (al-
tered balance and lethargy) within 3 weeks. MT330 tumors
 grew slowly for several weeks before reaching the lumines-
cence intensity and tumor volume (47 ± 14 mm\(^3\) as assessed
by MRI, as specified in the Supplementary Data, at week 15;
\( n = 5 \) mice) of U87 tumors.

**Permeability of the tumor vasculature**

We chose to characterize the BBB of our tumor models
using a CE-MRI approach. If the BBB were disrupted, CNS
drug penetration would be altered and it would be possible
to assess the effect of ABC transporter inhibitors at the level
of tumor cells (not tumor vessel). Using CE-MRI, we char-
acterized the vascular integrity of both U87 and MT330, and
images showed significant contrast enhancement, which
confirmed an impaired BBB (Supplementary Fig. S1A).
Homogeneous enhancement was observed in U87 tumors,
significantly greater than enhancement in the contralateral
brain tissue (Supplementary Fig. S1B). Contrast in MT330
tumors was inhomogeneous, with areas of high signal intensity
that may have indicated heavy vascularization and/or
necrosis.

**Topotecan penetration of tECF**

We anticipated greater drug concentrations within U87 and
MT330 tECF than in normal brain tissue ECF because of the
greater vascular permeability of the tumors. Our previous mi-
crodialysis studies showed the exposure of brain ECF to un-
bound topotecan lactone to be 21% to 36% that of plasma
after a 4 mg/kg i.v. bolus injection in FVB mice (11, 13). Here,
using an identical experimental setup, we first evaluated brain
ECF penetration in nontumor-bearing CD1 nude mice; results
were within the range previously reported (\( P_{\text{brain}} = 0.30 \pm
0.11, n = 7 \)). We then compared tECF versus contralateral
brain ECF penetration in animals bearing U87 and MT330
tumors. Unbound drug penetration of U87 tECF (\( P_{\text{tumor}} = 0.96 \pm
0.25, n = 7 \)) was 2.3 times that of contralateral brain
eCF (\( P_{\text{contralateral}} = 0.42 \pm 0.11, n = 5 \); \( t \) test; Fig. 2A).

In MT330 tumors, \( P_{\text{tumor}} (0.78 \pm 0.26, n = 6) \) and \( P_{\text{contralateral}}
(0.42 \pm 0.11, n = 5) \) also differed significantly (2.2-fold;
\( t \) test). \( P_{\text{contralateral}} \) values did not differ statistically
from \( P_{\text{brain}} \) values in the nontumor-bearing mice. Tumor
targeting (% experiments with probe in tumor) was more

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**Figure 2.** Topotecan penetration of tumor and contralateral brain ECF after a 4 mg/kg i.v. dose in U87 and
MT330 models. A, comparison of \( P_{\text{tumor}} \) means (SD), calculated
as \( \frac{\text{AUC}_{\text{tumorECF}}}{\text{AUC}_{\text{plasma}}} \)

\( t \) test, compared with contralateral
brain. B, representative
concentration-time graphs
(experimental data and
model-fitted curves) of topotecan
(unbound lactone) in ECF and
plasma. Histologic images
confirming probe location are
shown (yellow dots limit tumor
margins, blue dashes limit cannula
track, and red dashes limit probe
track; lines were drawn with
Adobe Photoshop V11.0).
accurate in the U87 model (88%) than in the MT330 model (55%). Figure 2B shows representative experiments.

Expression of ABC transporters in tumor lines and xenografts

Because one of our objectives was to evaluate the effect of inhibition of tumor cell ABC transporters on drug penetration, we examined the presence of these transporters by Western blot (in the cell lines) and immunohistochemistry (in the xenografts). As shown in Fig. 3A, BCRP, MRP1, and MRP4 were present in both tumor lines, whereas Pgp was not detected. In the U87 and MT330 xenografts, BCRP and MRP1 were strongly expressed, and Pgp and MRP4 were expressed moderately or weakly (Fig. 3B).

Gefitinib shifts intratumor topotecan distribution toward the intracellular compartment

To describe drug distribution in the tECF, vascular, and intracellular tumor compartments, we maintained a constant plasma drug concentration, determined the $C_{ss, tECF}/C_{ss, plasma}$ ratio ($P_{tumor}$), and estimated intratumor distribution ($V_{u,tumor}$) by using combined data sets obtained from microdialysis and homogenate samples. This strategy allowed us to (a) use fewer animals than studies using i.v. injections, which require serial tissue samples; (b) characterize the real-time effects of gefitinib on topotecan pharmacokinetics; and (c) achieve equivalent systemic exposures in animal groups that did and did not receive gefitinib.

We previously showed that gefitinib (likely through its interaction with ABC transporters) halves topotecan lactone plasma clearance (11, 13). In our steady-state studies, $C_{ss, plasma}$ (unbound lactone) was $34 \pm 8$ ng/mL ($n = 15$) after a $25 \mu g/h$ topotecan infusion but increased to $109 \pm 42$ ng/mL after administration of gefitinib ($n = 15; P < 0.001$). Figure 4A displays plasma unbound topotecan lactone concentration in all experiments in which gefitinib was administered 14 hours after topotecan pump insertion.

In the TPT12-GEF group ($n = 6$), which received $200 \text{ mg/kg}$ gefitinib before pump insertion, we corrected the topotecan dosage to $12.5 \mu g/h$ and achieved plasma exposure ($C_{ss, plasma}$ $33 \pm 2$ ng/mL) similar to that in animals receiving $25 \mu g/mL$ without gefitinib.

We used the U87 model to characterize intratumor drug distribution with the steady-state approach because these tumors are easily identified and dissected, grow faster and more consistently, and have an ABC transporter expression profile similar to that of MT330 tumors. We first performed microdialysis experiments to define topotecan $C_{ss,tECF}$. After a $25 \mu g/h$ topotecan infusion, the mean $C_{ss,tECF}$ was $22 \pm 10$ ng/mL ($n = 10$) after administration of gefitinib ($200 \text{ mg/kg}$; $P = 0.019$, paired t test). Figure 4B shows a representative experiment in a single animal.

Microdialysis experiments were also performed in the TPT12-GEF group, which received $12.5 \mu g/h$ topotecan after $200 \text{ mg/kg}$ gefitinib. $C_{ss,tECF}$ ($6.4 \pm 0.8$ ng/mL, $n = 3$) was significantly lower than that in animals that had not received gefitinib ($P = 0.014$, Mann-Whitney test).

When $P_{tumor}$ (i.e., $C_{ss,tECF}/C_{ss,plasma}$ ratio) was calculated, we observed lower ratios in both groups of animals receiving gefitinib than in the TPT25 group (without gefitinib; Fig. 4C). We hypothesized that gefitinib might have depleted...
topotecan lactone from the tECF and induced drug accumulation in the intracellular compartment, likely by inhibiting ABC transporters in the tumor cells.

To test our hypothesis, we calculated the volume of distribution of unbound drug in tumor ($V_{u,tumor}$) to describe intratumor drug distribution in vivo. New studies in U87 tumor-bearing mice provided the total (bound + unbound) topotecan lactone concentration at the steady-state in tumor homogenates ($C_{tot,tumor}$, 47 ± 21, 142 ± 67, and 43 ± 17 ng/mg tumor) and blood ($C_{tot,blood}$, 105 ± 33, 219 ± 81, and 109 ± 8 ng/ml) of the TPT25 ($n = 8$), TPT25-GEF ($n = 7$), and TPT12-GEF ($n = 6$) groups, respectively. The mean unbound topotecan tECF concentrations ($C_{u,ECF}$) from the microdialysis experiments were then used to calculate $V_{u,tumor}$. As shown in Fig. 5A, $V_{u,tumor}$ was significantly higher in groups receiving gefitinib. Thus, after administration of gefitinib, a greater proportion of drug was intracellular or nonspecifically bound to tumor tissue components rather than as unbound drug in the tECF.

To further characterize intracellular topotecan accumulation in the presence of gefitinib, we incubated U87 and MT330 cells with topotecan (0.1 μmol/L) and gefitinib (1 or 10 μmol/L). Topotecan accumulation was enhanced as much as 36% by gefitinib in both U87 and MT330 cells in a dose-dependent manner (Fig. 5B). This finding supports the shift of topotecan distribution toward the intracellular compartment in the presence of gefitinib.

**Effect of gefitinib on tumor cell sensitivity to topotecan**

To assess whether gefitinib sensitizes tumor cells to topotecan, we measured the growth of U87 and MT330 cells in the presence of topotecan, topotecan plus gefitinib (1 or 10 μmol/L), and gefitinib alone (Fig. 6). Gefitinib alone (0.001–50 μmol/L) did not substantially affect growth, but the IC$_{50}$ values of topotecan decreased in both cell lines with the addition of 10 μmol/L gefitinib [U87: from 15.1 (12.3–17.8) μmol/L to 3.54 (2.73–4.34) μmol/L; MT330: from 4.81 (3.84–5.79) μmol/L to 3.27 (2.71–3.83) μmol/L; means and 95% confidence intervals are provided].

**Discussion**

The ability of selected TKIs to enhance drug penetration of tumors by inhibiting ABC transporters in vivo is not completely understood. Here, we used two glioma models—a cell line (U87) and a primary culture (MT330)—to assess the effect of the TKI gefitinib on the tumor penetration and intratumor distribution of topotecan both in vitro and in orthotopic xenografts in CD1 athymic nude mice. Our results show that the pharmacologic interaction of the drugs results in increased intracellular tumor exposure to topotecan. The experimental model we used provides a novel method of assessing the effect of a modulator of drug efflux on the intratumor distribution of a chemotherapy agent in orthopaically engrafted brain tumors.

Our previous studies of topotecan-gefitinib and irinotecan-gefitinib combinations in transporter-overexpressing cells (6, 14, 35) and in brain penetration analyses (11, 13) showed that gefitinib inhibits at least two topotecan transporters: BCRP and Pgp. The inhibition of these two transporters in brain tumors could affect drug efflux at two levels: BBB vessels and tumor cells. We previously showed in an intact BBB...
model that gefitinib enhances drug penetration of the BBB (11, 13); in the present study, we focused on its effect at the tumor cell level. Therefore, we selected tumor models (U87 and MT330) that show increased vascular permeability (altered BBB) and ABC transporter expression. The U87 tumor vasculature does express multidrug resistance proteins like Pgp that may act as drug efflux pumps in intracerebral tumor models (23, 40). However, our contrast MRI (Supplementary Fig. S1) and microdialysis (Fig. 2) results, which confirm the increased permeability of the U87 tumor vessels, call into question the activity of this or other putative transporters at the tumor cell level. At the cellular level, we found abundant BCRP expression in U87 and MT330 lines, consistent with clinical findings in gliomas (41). Topotecan is also a substrate of MRP4 (35) and likely of MRP1 (42), both of which we found in U87 and MT330 tumor lines (Fig. 3). To our knowledge, gefitinib inhibition of MRP4 and MRP1 has not been studied in detail, although a similar compound, erlotinib, did not alter MRP1-related drug resistance (17). Therefore, the activity of MRP1 and MRP4 in our tumor lines may at least partially explain their relatively high resistance to topotecan even in the presence of gefitinib.

The steady-state experimental approach allowed us to observe the real-time effects of gefitinib on the topotecan steady-state ECF-to-plasma concentration ratio in individual animals. The decrease in this ratio after gefitinib administration was confirmed in a group of gefitinib-exposed animals (TPT12-GEF) whose topotecan dose was reduced to achieve systemic exposure equivalent to that achieved without gefitinib at a higher dosage (25 μg/h; equivalent plasma exposure helped to minimize the effect in which higher plasma levels can increase drug penetration of the intracellular compartment). These observations prompted us to investigate whether inhibition of transporters at the tumor cell level would drive drug distribution toward the tumor intracellular compartment. Data from our in vivo studies of homogenate-microdialysis concentration and from our in vitro studies of intracellular accumulation supported this shift in topotecan intratumor distribution. We did not evaluate intratumor gefitinib concentration, although good penetration was reported in subcutaneous glioma xenografts (gefitinib concentration >20 μmol/L for 8 h after a single oral dose of 55 mg/kg; ref. 43). In patients with gliomas who received gefitinib (500 mg, orally for 5 d) before tumor resection, gefitinib Css was 7 to 25 μmol/L in the tumor homogenate (44).

We attempted the steady-state experimental approach to study the effect of gefitinib on topotecan CNS penetration in nontumor-bearing mice. However, we did not obtain interpretable data from this experiment because the intact BBB limited CNS drug penetration in a way that, with our bioanalytic system, we could not detect topotecan in the ECF dialysates collected before the administration of gefitinib.

Figure 5. Effect of gefitinib on topotecan accumulation in tumor xenografts in vivo and in tumor cells in vitro. A, V_{\text{tumor}} values at steady state in U87 tumors from mice receiving 25 μg/h topotecan (TPT25), 25 μg/h topotecan and 200 mg/kg gefitinib (TPT25-GEF), or 12.5 μg/h topotecan and 200 mg/kg gefitinib (TPT12-GEF; plasma exposure equivalent to that in the TPT25 group). Points, mean; bars, SD. *, P = 0.044; **, P = 0.002, one-way ANOVA, post hoc t test with Bonferroni correction. B, topotecan (TPT) accumulation in tumor cells in vitro in the presence and absence of gefitinib. Values are the percentage of the maximum accumulation in control cells (0.1 μmol/L topotecan, no gefitinib) at each time point. Points, mean (n = 3); bars, SD. *, P < 0.05; **, P < 0.01, compared with accumulation in control cells at the same time point, one-way ANOVA, post hoc t test with Bonferroni correction.
A plausible reason for this limitation of the infusion approach is that slow drug infusions penetrate the intact CNS less efficiently compared with rapid bolus injections likely because the plasma concentration driving drug diffusion across the BBB is much lower in the case of the infusions (45). Increasing the infusion rate to achieve elevated plasma $C_{ss}$ in the nontumor-bearing animals might have rendered detectable drug levels in the ECF, but the toxicity of topotecan at such increased plasma $C_{ss}$ precluded performing this study.

We suggest that microdialysis alone is only partially informative in brain tumor drug penetration studies because (a) high drug concentrations in tECF would be expected, given the usually altered BBB, and (b) only drugs in the tECF are analyzed, whereas most chemotherapy agents have intracellular targets. Further, microdialysis measurement of drug concentration in intracranial tumors is complex (40, 46, 47). Microdialysis was recently used elegantly to identify very high methotrexate levels in the tECF of patients’ highly contrasting gliomas, but as mentioned above, these levels do not reveal the tumor cell penetration of the drug (46). New techniques like the one proposed by Langer and colleagues (48), combining positron emission tomography and microdialysis, may overcome the limitations of microdialysis alone and allow assessment of intracellular drug penetration in unresected human tumors.

To conclude, ours is the first report characterizing the role of drug efflux inhibitors in shifting the intratumor distribution of substrate drugs toward the cellular compartment in an orthotopic glioma model. Specific sampling and analysis methods are necessary to assess the intratumor distribution of anticancer drugs. Future studies to bring these concepts into the preclinical and clinical fields are warranted. One example is the use of irinotecan in combination with the antiangiogenic agent bevacizumab for recurrent glioma (49). As prolonged antiangiogenic therapy is reportedly associated with restoration of the BBB (50), future studies should address how the possible recovery of the BBB and inhibitors of drug efflux affect the penetration of brain tumors by irinotecan.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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